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# Nuclear relocalisation of cytoplasmic poly(A)-binding proteins PABP1 and PABP4 in response to UV irradiation reveals mRNA-dependent export of metazoan PABPs

Hannah M. Burgess<sup>1,2,\*</sup>, William A. Richardson<sup>1</sup>, Ross C. Anderson<sup>1</sup>, Christine Salaun<sup>3,‡</sup>, Sheila V. Graham<sup>3</sup> and Nicola K. Gray<sup>1,2,§</sup>

<sup>1</sup>MRC Centre for Reproductive Health/MRC Human Reproductive Sciences Unit, Queens Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

<sup>2</sup>MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

<sup>3</sup>MRC-University of Glasgow Centre for Virus Research, Institute of Infection, Immunity, and Inflammation, College of Medicine, Veterinary Medicine and Life Sciences, Garscube Campus, University of Glasgow, Glasgow G61 1QH, UK

\*Present address: Department of Microbiology, NYU Langone Medical Center, 550 First Avenue, New York, NY 10016, USA

‡Present address: Centre de Recherche INSERM U845, Université Paris Descartes, Faculté de Médecine, 156 Rue de Vaugirard, F-75015 Paris, France

§Author for correspondence (Nicola.Gray@ed.ac.uk)

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## Summary

Poly(A)-binding protein 1 (PABP1) has a fundamental role in the regulation of mRNA translation and stability, both of which are crucial for a wide variety of cellular processes. Although generally a diffuse cytoplasmic protein, it can be found in discrete foci such as stress and neuronal granules. Mammals encode several additional cytoplasmic PABPs that remain poorly characterised, and with the exception of PABP4, appear to be restricted in their expression to a small number of cell types. We have found that PABP4, similarly to PABP1, is a diffusely cytoplasmic protein that can be localised to stress granules. However, UV exposure unexpectedly relocalised both proteins to the nucleus. Nuclear relocalisation of PABPs was accompanied by a reduction in protein synthesis but was not linked to apoptosis. In examining the mechanism of PABP relocalisation, we found that it was related to a change in the distribution of poly(A) RNA within cells. Further investigation revealed that this change in RNA distribution was not affected by PABP knockdown but that perturbations that block mRNA export recapitulate PABP relocalisation. Our results support a model in which nuclear export of PABPs is dependent on ongoing mRNA export, and that a block in this process following UV exposure leads to accumulation of cytoplasmic PABPs in the nucleus. These data also provide mechanistic insight into reports that transcriptional inhibitors and expression of certain viral proteins cause relocation of PABP to the nucleus.

**Key words:** Translation initiation factor, Translational reprogramming, Subcellular localisation, Cellular stress response, ICP27, PABPC4

## Introduction

Gene expression is regulated at many levels including transcription, mRNA processing, mRNA translation and mRNA stability, by complex and interrelated regulatory networks. Within the cytoplasm, reprogramming of translation is crucial for a wide variety of biological processes including early development, synaptic responses by neurons and adaptation to cell stress (reviewed by Richter, 2007; Spriggs et al., 2010). This is often achieved by changes in the activity of key proteins by phosphorylation (e.g. eIF2) or binding of regulatory factors (e.g. 4EBP). However, it is becoming increasingly clear that the subcellular localisation of basal translation or regulatory factors, as well as mRNAs, can play an important role in determining mRNA fate.

Metazoans encode a nuclear poly(A)-binding protein, PABPN1, and one or more cytoplasmic poly(A)-binding proteins (PABPs), with distinct functions within these cellular compartments. The best characterised cytoplasmic PABP, PABP1 (also known as PABPC1), has multiple roles in mRNA translation and stability (reviewed by Burgess and Gray, 2010; Kuhn and Wahle, 2004; Mangus et al., 2003), indicating it is a central regulator of gene expression. Consistent with this, PABP1 is essential for vertebrate development (Gorgoni et al., 2011) and multiple post-transcriptional mechanisms have evolved to control both its levels and availability within cells (reviewed by Derry et al., 2006; Gorgoni and Gray, 2004). Bound to the polyadenylated [poly(A)] tail of mRNAs, PABP1 interacts with eukaryotic initiation factor 4G (eIF4G), part of the eIF4F complex which is associated with the 5' cap structure. By bridging both ends of the mRNA to form a 'closed loop', PABP1 is thought to promote translation initiation by enhancing ribosome subunit recruitment (reviewed by Gorgoni and Gray, 2004; Mangus et al., 2003).

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Additional interactions of PABP1 with eIF4B and PABP-interacting protein 1 (PAIP1), which are associated with the 5' end of the message, are postulated to stabilise this structure (reviewed by Gorgoni and Gray, 2004; Mangus et al., 2003). The formation of this 'end-to-end' complex also protects mRNAs from degradation (reviewed by Kuhn and Wahle, 2004; Mangus et al., 2003). Although PABP1 generally has a protective effect on the poly(A) tail, it also plays a positive role in regulating deadenylation through interactions with components of deadenylation complexes, PAN3 (Uchida et al., 2004) and TOB (Ezzeddine et al., 2007; Okochi et al., 2005) and the miRNA-induced silencing complex (miRISC)-associated protein GW182 (reviewed by Tritschler et al., 2010). Furthermore, by interacting with eukaryotic release factor 3 (eRF3), PABP1 promotes translation termination and prevents nonsense-mediated decay of messages that do not possess premature termination codons (Behm-Ansmant et al., 2007; Ivanov et al., 2008).

Although PABP1 is diffusely cytoplasmic in normally proliferating cells, it is also found at sites of localised translation, such as neuronal dendrites (Wang et al., 2002), and is enriched at the leading edges of migrating fibroblasts (Woods et al., 2002). In conditions of cell stress, such as osmotic shock, PABP1 is incorporated into cytoplasmic stress granules (SGs) (Kedersha et al., 1999). SGs contain stalled translation initiation complexes and are composed of mRNA, small ribosomal subunits, specific initiation factors and numerous RNA-binding proteins, and are thought to facilitate storage and remodelling of mRNPs (reviewed by Anderson and Kedersha, 2009; Buchan and Parker, 2009). However, the function of PABP1 in stress granules or its role in their assembly has not been investigated.

Mammalian PABP1, although predominantly cytoplasmic, shuttles between the cytoplasm and the nucleus (Afonina et al., 1998). Overexpression of PABP1 in human cells leads to its accumulation in the nucleus (Afonina et al., 1998) indicating that its nuclear export, but not import, is limiting. In *Saccharomyces cerevisiae*, partially redundant RNA-dependent and -independent nuclear export pathways have been identified for the PABP1 homologue, Pab1 (Brune et al., 2005), which appears to serve nuclear as well as cytoplasmic roles. RNA-dependent nuclear export of PABP in *Schizosaccharomyces pombe* has also been observed (Thakurta et al., 2002). However, no classical import or export signals have been identified in mammalian PABPs, although RNA-independent export pathways appear to exist (Khacho et al., 2008; Woods et al., 2005).

The abundance of PABP1 in the cytoplasm is manipulated by several viruses. PABP1 is proteolytically cleaved during infection by lentiviruses, picornaviruses and caliciviruses (reviewed by Smith and Gray, 2010) separating its N-terminal region that contains multiple RNA-recognition motifs (RRMs) from its C-terminus that is composed of a proline-rich region and the PABC domain. Alteration of the nucleo-cytoplasmic distribution of PABP1 is now emerging as an alternative consequence of viral infection and, similar to PABP1 cleavage, is suggested to provide a mechanism to limit the translation of host mRNAs (Smith and Gray, 2010). Members of the Herpesviridae, Reoviridae and the Bunyaviridae families have been reported to relocalise PABP1 to the nucleus upon infection (Smith and Gray, 2010). In Kaposi's sarcoma-associated herpesvirus (KSHV) and rotavirus (Reoviridae), specific viral proteins have been identified as necessary for PABP relocalisation (Smith and Gray, 2010).

However, the mechanisms by which they achieve relocalisation await further definition.

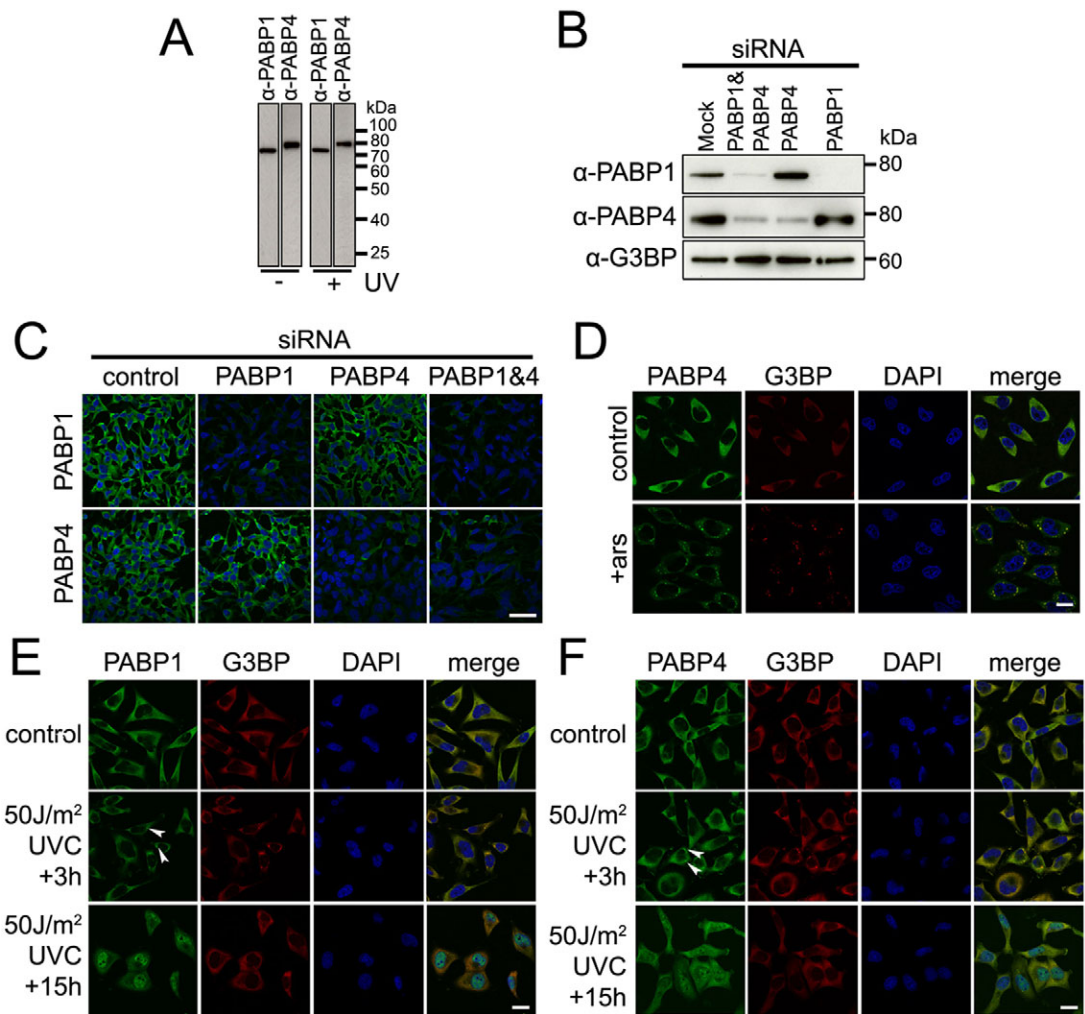
Although most studies to date have exclusively considered PABP1, mammals encode four cytoplasmic PABPs, which share a common domain organisation (reviewed by Gorgoni and Gray, 2004). Available data suggest that mRNAs encoding PABP1 and PABP4 (also known as iPABP and PABPC4) are widely expressed (Yang et al., 1995), whereas other members [embryonic PABP (ePABP) also called ePAB or PABP1L and testis-specific PABP (tPABP) also known as PABPC2 or PABPC3] appear restricted to germ cells and early embryos (reviewed by Brook et al., 2009). PABP4 is highly similar to PABP1 at the protein level, sharing 75% identity and binding poly(A) with a similar affinity to PABP1 (Sladic et al., 2004), suggesting that it might function analogously in the regulation of global mRNA translation and stability. Consistent with this, epitope-tagged PABP4 is predominantly cytoplasmic (Yang et al., 1995). Furthermore, the PABC domain, also known as the MLE domain, which mediates PABP1 contacts with PAM2 motif-containing proteins PAIP1, TOB, PAN3, GW182 and eRF3 (Albrecht and Lengauer, 2004) is highly conserved in PABP4 and accordingly interactions with eRF3 and TOB have been reported (Cosson et al., 2002; Okochi et al., 2005).

Here, we have examined the subcellular localisation of PABP1 and PABP4 in mammalian cells. This showed that endogenous PABP4 is a diffusely cytoplasmic protein that can be relocalised to SGs. Interestingly we found that although UV is a poor inducer of PABP relocalisation to SGs, it potently induces relocalisation of PABP1 and PABP4 to the nucleus. In exploring the mechanism of PABP relocalisation, we found a similar accumulation of poly(A) RNA in the nucleus following UV treatment. Neither mRNA export nor relocalisation was found to be dependent on PABP1 or PABP4. However, we found that blocking mRNA export recapitulated the nuclear accumulation of PABPs. Thus, nuclear export of PABP1 and PABP4 is, at least partially, dependent on active mRNA export, explaining their nuclear accumulation after UV irradiation, treatment with transcriptional inhibitors and exogenous expression of the herpes simplex virus-1 (HSV-1) protein ICP27.

## Results

### PABP1 and PABP4 relocalise to stress granules and to the nucleus after UV irradiation

Subcellular changes in the localisation of PABP1 are emerging as a common response to viral infection and cellular stress (Kedersha et al., 1999; Ma et al., 2009; Smith and Gray, 2010). However, many mammalian cell types are also likely to contain significant quantities of PABP4 (Katzenellenbogen et al., 2010; Yang et al., 1995) that will contribute to the overall subcellular distribution of PABP proteins. Thus we generated antibodies against PABP4 (and PABP1) to examine its localisation. Western blotting showed that each detected a single protein in HeLa cell extracts (Fig. 1A), with quantitative analysis indicating that PABP1 is approximately six times more abundant than PABP4 (supplementary material Fig. S1). Importantly, PABP1 was barely detectable in extracts from HeLa cells transfected with PABP1 small interfering RNA (siRNA), or both PABP1 and PABP4 siRNAs (Fig. 1B) and similarly PABP4 was only significantly reduced in cells transfected with the PABP4 siRNA (Fig. 1B), confirming the specificity of these newly generated antibodies. Consequently, these antibodies were



**Fig. 1. PABP1 and PABP4 relocalise to SGs early and the nucleus late after UV irradiation.** (A) PABP1 and PABP4 antibodies each detect a single protein of the expected size in extracts prepared from untreated HeLa cells or cells 15 hours after treatment with 50 J/m<sup>2</sup> UV. (B,C) Specificity of the PABP1 and PABP4 antibodies was demonstrated by western blotting (B) and immunofluorescence (C) following siRNA knockdown in HeLa cells: PABP1 and PABP4 levels were only reduced in response to their respective siRNAs. G3BP served as a loading control. Scale bar: 50 μm. (D) HeLa cells were fixed following exposure to 0.5 mM arsenite treatment or vehicle control (PBS; control) for 1 hour. G3BP (red) and PABP4 (green) were detected by immunofluorescence. (E,F) HeLa cells were fixed at 3 or 15 hours after UV treatment (50 J/m<sup>2</sup> 254 nm) or 15 hours after mock treatment (control). G3BP (red) and PABP1 or PABP4 (green) were detected by immunofluorescence. Arrowheads indicate UV-induced SGs. Scale bars: 20 μm. In C–F, DNA was stained with DAPI (blue).

applied to fixed cells treated with PABP-specific or control siRNAs to determine the subcellular localisation of PABP4. Importantly, this revealed that PABP4, like PABP1, is a predominantly cytoplasmic protein that appears diffusely distributed in normally proliferating cells (Fig. 1C). siRNA knockdown demonstrated the specificity of both antibodies for immunofluorescence (Fig. 1C). Because PABP1 is a well-known stress granule protein (Kedersha et al., 1999), we investigated whether PABP4 relocalises to SGs using treatments including arsenite (Fig. 1), which induces abundant large SGs. Ras-GAP SH3 domain binding protein (G3BP) was used as a marker of stress granules and PABP4 was found to move into G3BP-positive foci upon arsenite treatment (Fig. 1D), demonstrating that it is a component of stress granules. Consistently, PABP1 and PABP4 also localised to the small stress granules induced in a minority of cells (~5%), 3 hours after UV treatment (50 J/m<sup>2</sup>, 254 nm; Fig. 1E,F). However, unexpectedly

both PABP1 and PABP4 showed substantial relocalisation to the nucleus, in the majority of cells, later (15 hours) after UV irradiation (Fig. 1E,F). Relocalisation was partial, with both PABP1 and PABP4 still detectable in the cytoplasm, and western blotting of UV-treated cell extracts ruling out the possibility that the nuclear signal was due to the induction of cross-reacting nuclear proteins (Fig. 1A). The minimum dose required to relocalise PABP1 and PABP4 was 20 J/m<sup>2</sup>, which resulted in 85% of cells showing relocalisation, whereas doses over 30 J/m<sup>2</sup> relocalised PABP in more than 95% of cells. 10 J/m<sup>2</sup> did not result in significant relocalisation (supplementary material Table S1).

#### Apoptosis is not a requirement for UV-induced relocalisation of PABPs

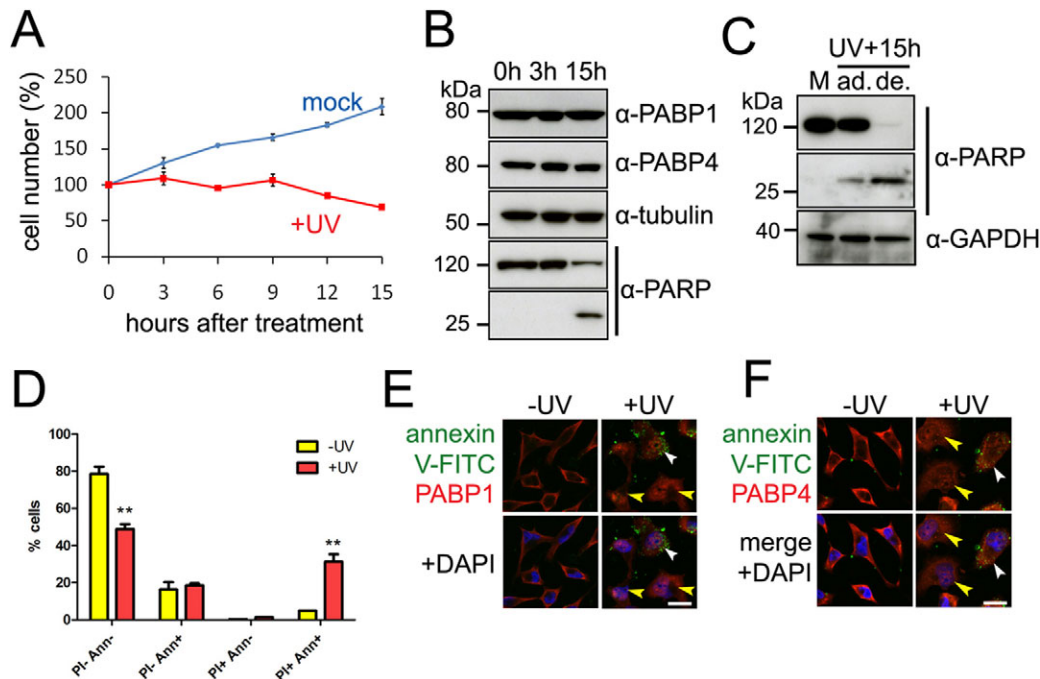
UV exposure results in two predominant types of DNA damage, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) (Batista et al., 2009). The



presence of DNA damage was confirmed in cells treated with  $50 \text{ J/m}^2$  UV using anti-CPD antibodies with lesions persisting at 15 hours (supplementary material Fig. S2A,B), consistent with studies showing that CPD repair can take 36 hours in this cell line (Powley et al., 2009). By contrast, significant removal of 6-4PP lesions was observed even at early time points, in keeping with the faster kinetics of 6-4PP repair (supplementary material Fig. S2C). DNA damage, following UV exposure, can result in cell growth arrest while repair occurs and/or cell death if damage is irreparable (Batista et al., 2009). In keeping with this, UV exposure resulted in the inhibition of adherent cell proliferation and a reduction in cell number at late time points (Fig. 2A). This reduction in cell number raises the possibility that PABP relocalisation is dependent on apoptosis, and intriguingly HSV-1 infection, which results in PABP1 nuclear relocalisation (Dobrikova et al., 2010; Salaun et al., 2010), also induces early stages of apoptosis, although cell death is prevented by the action of viral proteins (Nguyen and Blaho, 2007). Thus to assess the level of apoptosis in UV-treated cells, extracts were prepared from HeLa cells, including non-adherent cells in the culture medium 0, 3 and 15 hours after UV irradiation. Extracts were analysed by western blotting for poly(ADP-ribose) polymerase (PARP), which is cleaved by caspases during late apoptosis (Fig. 2B). A 24 kDa cleavage fragment was detected at 15 hours after UV treatment, but not at 0 or 3 hours, indicating that at

15 hours post-treatment, when PABP1 and PABP4 are relocalised, apoptosis is occurring. PABP1 has been reported to be degraded during apoptosis induced by cisplatin (Marissen et al., 2004); however, western blots of PABP1 and PABP4 showed no depletion of the intact proteins after UV exposure (Fig. 2B), and cleavage fragments were not detected (Fig. 1A).

Because we examined the localisation of PABPs only in the cell population that remains adherent (Fig. 1E,F), the degree of PARP cleavage in the adherent and floating cell populations 15 hours after UV exposure was examined separately (Fig. 2C). This revealed that the majority of cleaved PARP was present in the floating cell population, with comparatively little detected in the cells that remained adherent. To quantify the proportion of adherent cells undergoing apoptosis 15 hours after UV treatment, flow cytometry was used in conjunction with annexin V-FITC and propidium iodide (PI) staining (Fig. 2D and supplementary material Fig. S3). Annexin V binds with high affinity to externalised phosphatidylserine on the outer plasma membrane leaflet, a feature of early apoptosis, and PI can only penetrate and stain dead or dying cells. In untreated cells, 78.6% of cells were unstained by either marker and therefore viable. Annexin V alone stained 16.3% of cells, indicating early apoptosis, and 4.9% of cells stained for both annexin V and PI, indicating non-viability, due to late apoptosis or necrosis. Thus 21.2% of untreated cells were positive for annexin V and potentially apoptotic, with



**Fig. 2. Relocalisation of PABPs is independent of UV-induced apoptosis.** (A) Adherent HeLa cells were harvested at the indicated time following treatment with  $50 \text{ J/m}^2$  UV (red) or mock treatment (blue) and cell numbers were determined by flow cytometry. The number of cells at time zero was set to 100%; values are the means  $\pm$  s.e.m. of three independent experiments. (B) HeLa cell extracts were prepared from total (adherent and detached) cells 0, 3 or 15 hours after UV treatment ( $50 \text{ J/m}^2$  254 nm) and western blotted with the indicated antibodies. Tubulin was used as a loading control. (C) HeLa cell extracts were prepared from adherent cells 15 hours after mock treatment (M), or from adherent (ad.) or detached (de.) cells 15 hours after UV treatment ( $50 \text{ J/m}^2$ , 254 nm) and western blotted with the indicated antibodies. (D) Adherent cells were harvested 15 hours after either UV treatment ( $50 \text{ J/m}^2$  254 nm) or mock treatment and assayed for induction of apoptosis by flow cytometry (supplementary material Fig. S3) using annexin V-FITC and PI staining. Yellow bars: mock treated (-UV) cells; red bars: UV-treated (UV+) cells. Values are means  $\pm$  s.e.m. of two independent experiments;  $**P < 0.01$ . (E,F) HeLa cells were fixed 15 hours after UV treatment ( $50 \text{ J/m}^2$  254 nm) or mock treatment and annexin V-FITC (green) was used to stain apoptotic cells, visible as decoration around the cell membrane. PABP1 or PABP4 (red) were detected by immunofluorescence. DNA was stained with DAPI (blue). Fields containing both annexin V-positive (white arrowheads) and -negative (yellow arrowheads) cells were chosen, in which PABP1 (E) or PABP4 (F) was relocalised to the nucleus. Scale bars: 20  $\mu\text{m}$ .

agitation during cell trypsinisation and collection likely to be a contributing factor. After treatment with UV 48.9% of cells were unstained and viable, a significantly lower percentage ( $P<0.01$ ) than observed in untreated cells. Surprisingly, there was no significant difference in the percentage of treated and untreated cells stained for annexin V alone. This indicates that cells induced to apoptose had probably progressed to a late stage, consistent with PARP cleavage in treated cells (Fig. 2C) and a significant increase ( $P<0.01$ ) in the percentage of cells stained by both annexin V and PI (Fig. 2D). Because PABP1 and PABP4 exhibit nuclear relocalisation in more than 95% of treated adherent cells compared with 0% of control cells (supplementary material Table S1), the increase in potentially apoptotic cells from 21.2% to 49.8% suggests that PABP relocalisation is not restricted to cells undergoing apoptosis. This was confirmed by immunofluorescence, which showed relocalised PABP1 and PABP4 in adherent cells that were annexin V negative (Fig. 2E,F). Hence apoptosis does not drive relocalisation.

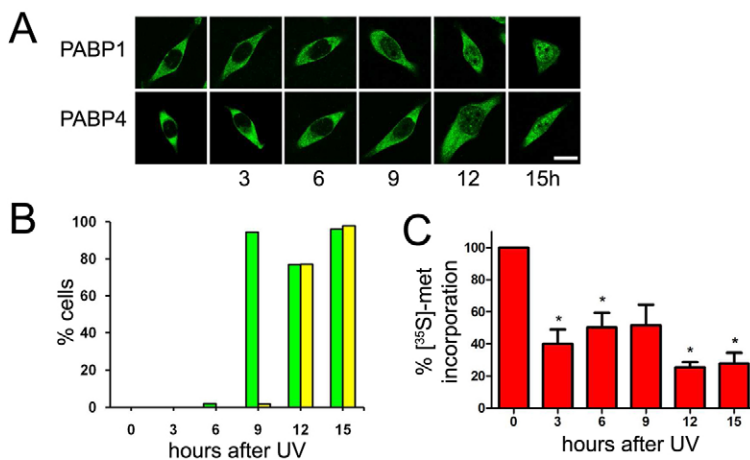
#### Decreased protein synthesis correlates with relocalisation of cytoplasmic PABPs

In the context of viral infection, relocalisation of PABP1 from the cytoplasm to the nucleus is proposed to inhibit cellular translation (reviewed by Smith and Gray, 2010). The timing of PABP1 and PABP4 relocalisation after UV treatment was therefore compared with protein synthesis rates. PABP1 was weakly visible within nuclei from 9 hours post-treatment with its nuclear intensity increasing at 12 and 15 hours (Fig. 3A), consistent with a steady accumulation of PABP1 in the nucleus over time. By 9 hours post-treatment, 94.4% of cells contained nuclear PABP1, although this decreased to 76.8% at 12 hours (Fig. 3B), perhaps reflecting a detachment of some cells containing PABP1 in the nucleus. Similarly, nuclear PABP4 increased between 12 and 15 hours post-treatment but was barely detectable above background at 9 hours, hinting at a difference in the rate of accumulation of the two proteins (Fig. 3A,B). Although the percentage of cells in which PABP1 and PABP4 relocalised was similar at the 12 and 15 hour time points (Fig. 3B), immunofluorescence showed that PABP4 was relocalised to a lesser extent (Fig. 3A). The rate of protein synthesis was measured in cells following UV treatment by monitoring [ $^{35}$ S]methionine incorporation (Fig. 3C). Rates, which were adjusted for changes in cell number, were

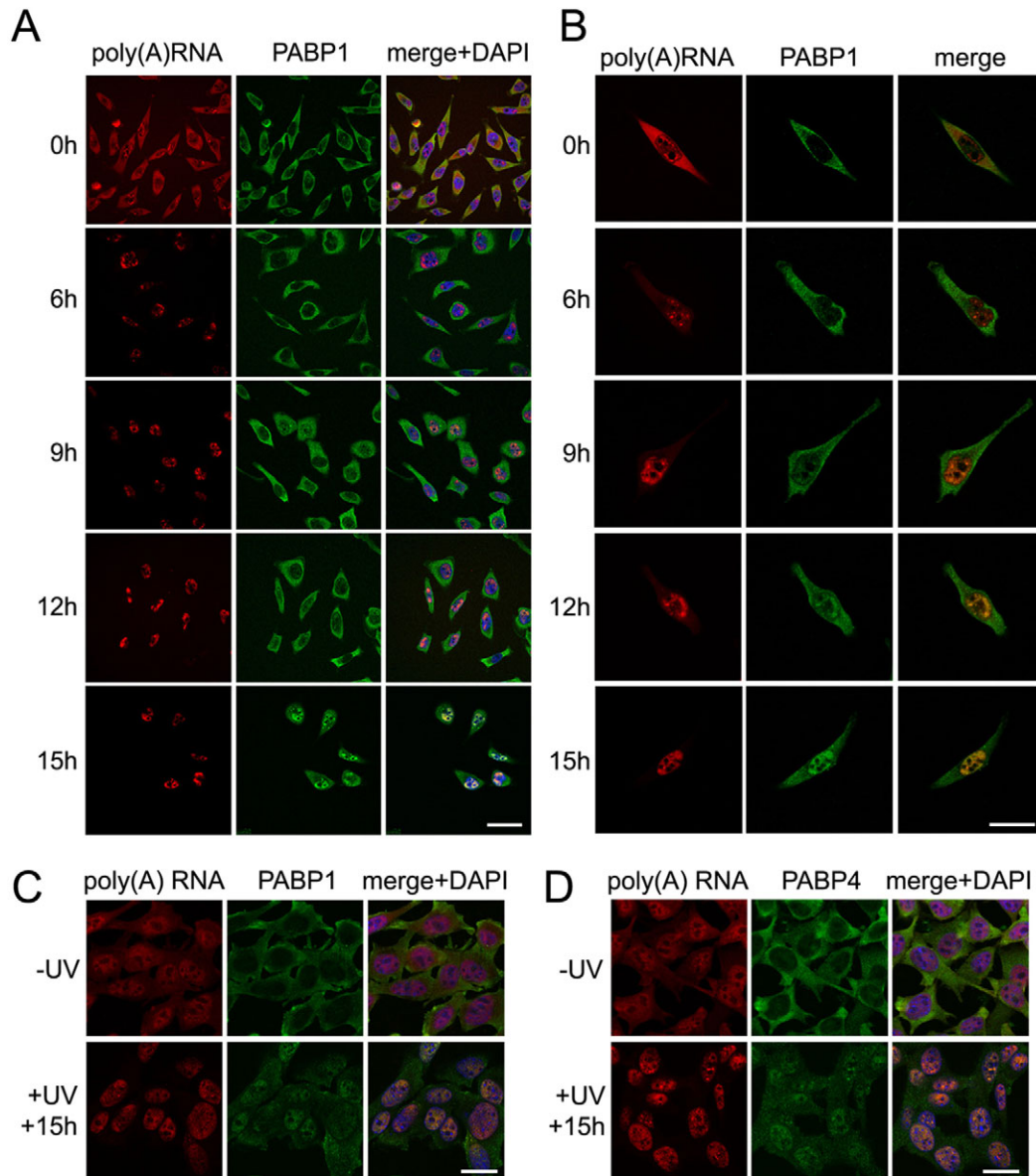
substantially reduced within 3 hours, dropping to 39.9% of the rate in untreated cells and remaining at ~40–50% at 6 and 9 hours post-treatment, consistent with the observations of others using the same UV dose (Wu et al., 2002). A further reduction of protein synthesis to 25–28% was observed between 9 and 12 hours. During this time PABP1 nuclear intensity increased dramatically, and PABP4 relocalisation became apparent (Fig. 3A,B). This raises the possibility that nuclear relocation of one or both of the cytoplasmic PABPs contributes to the inhibition of global translation and/or translational reprogramming following UV exposure.

#### Poly(A) RNA distribution within cells is dramatically altered following UV exposure

PABP nuclear export in metazoans is not well defined and thus we sought to determine the mechanism by which it is blocked following UV irradiation. Intriguingly, overexpressed PABP1 accumulates in splicing speckles (Afonina et al., 1998), which are enriched in poly(A) RNA (reviewed by Anderson and Kedersha, 2009; Lamond and Spector, 2003) and we observed a colocalisation of endogenous PABP1 and PABP4 with the splicing speckle marker SC-35 following UV exposure (supplementary material Fig. S4). Similarly, stress granules, which contain PABP1 and PABP4, are also enriched in poly(A) RNA (reviewed by Anderson and Kedersha, 2009; Lamond and Spector, 2003), and PABP and poly(A) RNA are both retained in the nucleus of mRNA export mutants in budding and fission yeast (Brune et al., 2005; Thakurta et al., 2002). This raises the possibility that the relocalisation of metazoan PABPs might be linked to poly(A) RNA distribution. To investigate this hypothesis, we utilised a Cy3-labelled oligo(dT)<sub>40</sub> probe to monitor poly(A) RNA localisation by fluorescence in situ hybridisation (FISH). As controls, HeLa cells were treated with RNase post-fixation to demonstrate the specificity of the probe for RNA, or with arsenite before fixation to show that changes in RNA localisation could be distinguished (supplementary material Fig. S5). Following UV treatment, cells were subject to oligo(dT) FISH and immunofluorescence analysis for PABP1 (Fig. 4) and the intensity of fluorescence across individual cells determined (supplementary material Fig. S6). Nuclear levels of poly(A) RNA were not significantly altered by 6 hours post-treatment, although the cytoplasmic FISH signal was significantly diminished, remaining low thereafter. Intriguingly, however, 9 hours after



**Fig. 3. PABP relocalisation is correlated with decreased protein synthesis.** (A) HeLa cells were fixed at 3-hour intervals after UV treatment ( $50 \text{ J/m}^2$  254 nm) or immediately after mock treatment (0 hours) and PABP1 or PABP4 detected by immunofluorescence. Scale bar: 20  $\mu\text{m}$ . (B) The frequency of detectable PABP1 (green bars) or PABP4 (yellow bars) in the nucleus was evaluated in more than 200 cells at each time point. Dividing cells were not counted. Three separate experiments were conducted with very similar results and the data from one such experiment are shown. (C) Protein synthesis rates in HeLa cells at 3-hour intervals after UV treatment ( $50 \text{ J/m}^2$  254 nm) was measured by [ $^{35}$ S]methionine incorporation. Each experiment was performed in triplicate and the means normalised to cells assayed immediately after mock treatment (0 hours) and expressed as a percentage. Values are means  $\pm$  s.e.m. of three independent experiments.



**Fig. 4. PABP relocalisation mirrors accumulation of poly(A) RNA in the nucleus after UV treatment in HeLa and NIH3T3 cells.** (A,B) HeLa cells were fixed at 3-hour intervals after UV treatment ( $50 \text{ J/m}^2$  254 nm) or after mock treatment (0h). Poly(A) RNA was detected by FISH using a Cy3-oligo(dT)<sub>40</sub> probe (red) and PABP1 (green) was detected by immunofluorescence analysis. Single representative cells are shown in B. (C,D) NIH3T3 cells were fixed 15 hours after UV treatment ( $50 \text{ J/m}^2$  254 nm) or mock treatment (–UV) and subject to immunofluorescence analysis as in A. DNA was stained with DAPI (blue). Scale bars: 50  $\mu\text{m}$  (A) and 20  $\mu\text{m}$  (B–D).

treatment, it was evident that poly(A) RNA had significantly increased in abundance in the nucleus (Fig. 4A,B and supplementary material Fig. S6), consistent with a block in mRNA export. Nuclear relocalisation of PABP1 occurs in the majority of cells by 9 hours (Fig. 3B) but the levels of PABP1 in nuclei continue to increase until 15 hours post-treatment (Fig. 3A, Fig. 4A,B and supplementary material Fig. S6) indicating that PABP relocalisation occurs with slower kinetics than nuclear poly(A) accumulation (see Discussion).

To determine whether these relocalisation events are recapitulated in other mammalian species and cell types, the same UV treatment was applied to the mouse fibroblast cell line NIH3T3 and the localisation of poly(A) RNA with Pabp1 or Pabp4 examined 15 hours later. As in HeLa cells, robust nuclear relocalisation of Pabp1 and Pabp4 was observed (Fig. 4C,D) and was accompanied by a restriction of poly(A) RNA to the nucleus. Thus a block in the nuclear export of both poly(A) RNA and cytoplasmic PABPs following UV exposure appears to be a general characteristic of mammalian cells.

#### PABP1 and PABP4 are not determinants of poly(A) RNA localisation

Although PABP1 and PABP4 are clearly able to enter the nucleus (Fig. 1), poly(A) tails in the nucleus are thought to be bound by PABPN1 in all studied eukaryotes, with the exception of *S. cerevisiae*. PABPN1, which has no significant sequence similarity to cytoplasmic PABPs, is involved in regulation of polyadenylation (Kuhn and Wahle, 2004; Mangus et al., 2003) and has been suggested to be important for mRNA export (Apponi et al., 2010; Calado et al., 2000). It is generally considered that the exchange of PABPN1 for PABPC1 on the poly(A) tail occurs after the nuclear export of mRNA (Lemay et al., 2010); however, PABP1 has been isolated bound to pre-mRNA from the nucleus of mammalian cells (Hosoda et al., 2006) and has been shown to accumulate in the nucleus following transcriptional inhibition (Afonina et al., 1998), which is coupled to mRNA export (Reed, 2003). This latter property is shared by PABP4, as it relocalises to the nucleus following prolonged treatment with a transcriptional inhibitor (supplementary material

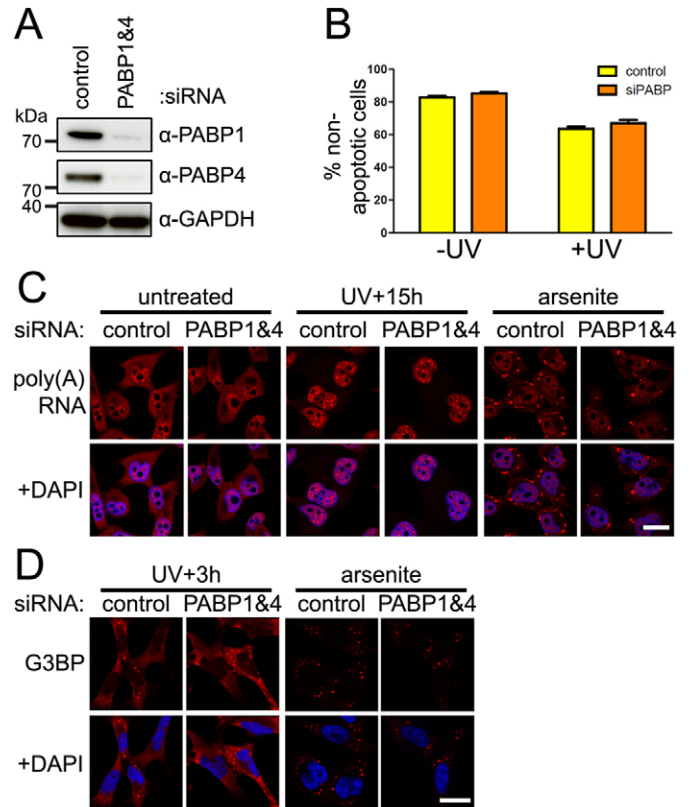


Fig. S7). Thus there is evidence to suggest that cytoplasmic PABPs can bind RNAs in the nucleus alongside PABPN1, implying that export of mammalian cytoplasmic PABPs could be dependent on ongoing mRNA export. If this hypothesis is correct, the nuclear accumulation of PABPs after UV exposure could result from the observed block in poly(A) RNA export (Fig. 4). However, it has recently been suggested that nuclear relocation of cytoplasmic PABPs affects the distribution of poly(A) RNA, perhaps by retaining it in the nucleus or impeding its export (Kumar and Glaunsinger, 2010).

We therefore tested whether PABPs are responsible for the changes in poly(A) RNA distribution observed after UV exposure by treating HeLa cells with both the PABP1- and PABP4-specific siRNAs that efficiently knockdown PABP proteins (Fig. 1). Efficient PABP knockdown was confirmed by western blotting (Fig. 5A), and shown not to influence cell viability following UV treatment (Fig. 5B). However, a reduction in cell number was noted in both untreated and UV-treated cells suggesting that cell growth and/or cell division could be retarded by a reduction in PABP levels (data not shown). Under normal growth conditions simultaneous knockdown of PABP1 and PABP4 did not affect the distribution of poly(A) RNA (Fig. 5C, left panel). This indicates that neither protein is essential for bulk mRNA export, consistent with experiments using deletion mutants in *S. cerevisiae* (Brune et al., 2005) and *S. pombe* (Thakurta et al., 2002). Importantly, after UV exposure the same restriction of poly(A) RNA to the nucleus was observed in control siRNA and PABP1/4 siRNA-treated cells (Fig. 5C, middle panel), indicating that the change in RNA distribution is not dependent on PABP1 or PABP4. Because the role of PABPs in SG assembly has not been assessed, we also examined SG formation in PABP knockdown cells, which revealed that poly(A) RNA was still recruited to SGs formed after arsenite treatment (Fig. 5C – right panel). Similarly, SGs were shown to be present after UV exposure (Fig. 5D). G3BP immunofluorescence was used, in this instance, because these SGs proved difficult to identify by FISH. Thus, PABPs do not affect the steady state localisation of poly(A) RNA or recruitment of RNA to SGs, nor is their presence in the nucleus required for the accumulation of nuclear poly(A) RNA after UV treatment.

#### A block in mRNA export recapitulates the nuclear accumulation of PABP1 and PABP4 observed after UV exposure

Transfection of the HSV-1 protein ICP27 relocates PABP1 to the nucleus (Dobrikova et al., 2010; Salaun et al., 2010), although ICP27 is not required for PABP1 relocalisation in the context of infection (Salaun et al., 2010). ICP27 is a nucleo-cytoplasmic shuttling protein that inhibits cellular splicing (Hardy and Sandri-Goldin, 1994) and promotes nuclear export of viral RNAs by recruiting the mRNA transport proteins, TAP and Aly/REF (Chen et al., 2005; Chen et al., 2002; Koffa et al., 2001). Because mRNA splicing promotes export (Valencia et al., 2008), inhibition of splicing by ectopically expressed ICP27 would be predicted to inhibit mRNA export. This effect might be further exacerbated by ICP27 sequestering TAP and Aly/REF and could underlie the nuclear relocalisation of PABP1. To test this hypothesis we transfected GFP-tagged ICP27 into HeLa cells and examined the localisation of poly(A) RNA, PABP1 and PABP4. In transfected cells, poly(A) RNA was strikingly restricted to the nucleus, and both PABP1 and PABP4 showed

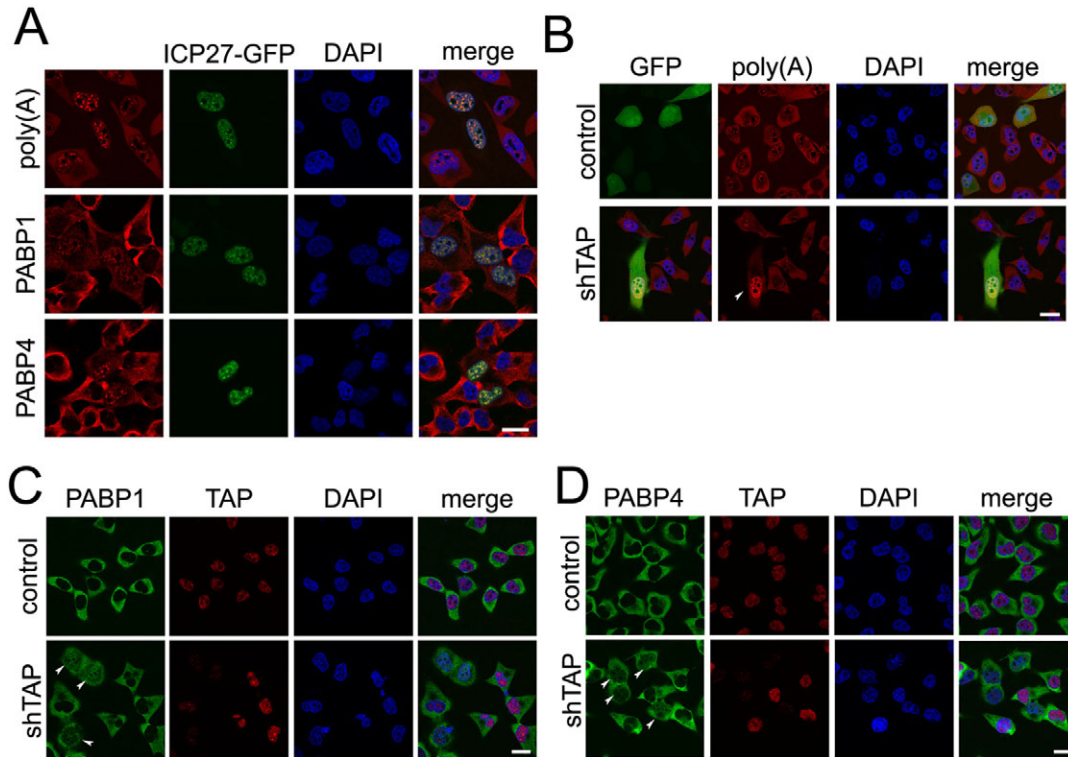


**Fig. 5. PABP1 and PABP4 are not determinants of poly(A) RNA localisation.** (A) Knockdown of PABP1 and PABP4 protein in siRNA-treated HeLa cells was confirmed by western blotting. GAPDH was used as a loading control. (B) After transfection with a control siRNA (yellow bars) or PABP1 and PABP4-specific siRNAs (orange bars), HeLa cells were treated with 50 J/m<sup>2</sup> UV followed by 15 hours incubation, and adherent cells assayed for annexin V-FITC and PI staining by flow cytometry. Percentage annexin V-negative and PI-negative cells are shown. Data represented as means  $\pm$  s.e.m. of three independent experiments. (C) Control or PABP1 and PABP4 siRNA-transfected HeLa cells were treated with 50 J/m<sup>2</sup> UV followed by 15 hours incubation or with 0.5 mM sodium arsenite for 1 hour. Poly(A) RNA was detected by FISH using a Cy3-oligo(dT)<sub>40</sub> probe (red). DNA was stained by DAPI (blue). Scale bar: 20  $\mu$ m. (D) siRNA-transfected HeLa cells were treated with 50 J/m<sup>2</sup> UV followed by 3 hours incubation or with 0.5 mM sodium arsenite for 1 hour. The cells were then fixed and G3BP (red) detected by immunofluorescence. DNA was stained with DAPI (blue). Scale bar: 20  $\mu$ m.

partial nuclear relocalisation (Fig. 6A), reminiscent of their localisation following UV exposure. Thus inhibition of transcription (supplementary material Fig. S7) or splicing (Fig. 6A), both of which affect mRNA export, leads to PABP1 and PABP4 redistribution.

To directly test whether mammalian cytoplasmic PABPs are exported via an mRNA-dependent pathway, we transfected cells with shTAP, which produces a short hairpin RNA that targets the bulk mRNA export adaptor TAP, blocking poly(A) RNA export (Williams et al., 2005). The shTAP construct was initially co-transfected with a GFP reporter to mark transfected cells, and FISH was used to confirm the effect of TAP RNAi on poly(A) RNA, which was restricted to the nucleus in transfected cells (Fig. 6B). Next, PABP1 and TAP were detected by immunofluorescence in shTAP-transfected cells. Crucially, in





**Fig. 6. PABP relocalises to the nucleus when mRNA export is blocked.** (A) HeLa cells were transfected with 350 ng/ml ICP27–GFP. 24 hours post-transfection, poly(A) RNA (top panel) was detected by FISH (red) and PABP1 and PABP4 (middle and bottom panels, respectively) detected by immunofluorescence (red). (B) HeLa cells were transfected with 3 µg shTAP or control plasmid and 1 µg GFP (pEGFP-C1). A cell transfected with shTAP is indicated by an arrowhead. 65 hours post-transfection poly(A) RNA was detected by FISH using a Cy3–oligo(dT)<sub>40</sub> probe (red). (C,D) HeLa cells were transfected with 3 µg shTAP or control plasmid and 65 hours post-transfection TAP (red) and PABP1 (C, green) or PABP4 (D, green) were detected by immunofluorescence. Arrowheads indicate cells in which TAP knockdown was efficient. DNA was stained with DAPI (blue). Scale bars: 20 µm.

cells in which TAP was depleted, PABP1 was relocalised to the nucleus (Fig. 6C). Similarly, PABP4 was also relocalised in TAP-depleted cells (Fig. 6D), consistent with the dependence of PABP1 and PABP4 nuclear export on active mRNA export.

Taken together our data support a model in which nuclear export of cytoplasmic PABPs is dependent on ongoing mRNA export, and that a UV-stress-induced block in this process can lead to accumulation of cytoplasmic PABPs in the nucleus.

## Discussion

In this study, we describe the subcellular distribution of PABP4 and the relocalisation of PABP1 and PABP4 to the nucleus of mammalian cells after UV irradiation. We found that relocalisation is preceded by an accumulation of poly(A) RNA in the nucleus, which is not dependent on PABPs, and reveal, by contrast, that the transit of cytoplasmic PABPs from the nucleus in metazoa is dependent on mRNA export.

Although PABP1 was initially visible in the nucleus at the time when the nuclear accumulation of poly(A) RNA was first apparent (9 hours), the relocalisation of the majority of PABP1 occurred with slower kinetics. The slower relocalisation of PABP1 is to be expected because the vast majority of PABP is cytoplasmic prior to UV treatment, and therefore its accumulation in the nucleus will be dependent on the pool of PABP that over time is cycled to the nucleus, but which becomes trapped and does not re-enter the cytoplasm because of the block in mRNA export. In keeping with a mRNA-dependent mode of

PABP1 export, the accumulation of PABP1 in the nucleus could be recapitulated by several perturbations, most notably by directly targeting the mRNA export factor TAP.

We consistently found that PABP4 behaved similarly to PABP1 in its localisation and export. However, our data also suggested that PABP4 nuclear relocalisation might be subtly retarded with respect to the kinetics of its movement and the extent of its relocalisation (Fig. 3). The basis of these differences is unclear but could be due to differences in their amino acid sequences affecting their post-translational modification status or their ability to interact with protein partners. The majority of the diversity between the two proteins is located in the proline-rich region that links the RRM and the PABC domain and which is predicted to be alternatively spliced in PABP4, raising the possibility that specific splice variants remain exclusively cytoplasmic. Sequence differences could also affect their respective RNA-binding capacity but PABP4 has been shown to bind poly(A) *in vitro* with a similar affinity to PABP1 (Sladic et al., 2004) and our finding that it is exported by a mRNA-dependent pathway provides support that it binds poly(A) tails *in vivo*.

The nuclear relocalisation of PABP1 and PABP4 did not appear to be linked to apoptosis (Fig. 2) and consistent with this PABP1 remains diffusely cytoplasmic following gamma irradiation-induced DNA damage (Salaun et al., 2010), which is known to induce apoptosis. Given the role of PABP1 in stimulating cellular translation (Derry et al., 2006; Gorgoni and

Gray, 2004; Mangus et al., 2003), the removal of a substantial proportion of PABP1 and PABP4 from the cytoplasm following UV treatment (Fig. 1) would be predicted to reduce global translation rates. Supporting this, PABP1 knockdown reduces global translation (supplementary material Fig. S10), which is consistent with other studies in HeLa cells that found a 15–50% loss of translation (Blakqori et al., 2009; Mokas et al., 2009; Yoshida et al., 2006). This raises the possibility that the reduction in protein synthesis rates between 9 and 12 hours (Fig. 3) could be attributable, at least in part, to the effect of PABP1 relocalisation. The absence of further reduction in translation rates between 12 and 15 hours might be an indication of PABP levels dropping beneath a threshold level. However, a firm conclusion regarding the effects of PABP relocalisation on global protein synthesis is made difficult by the complexity of effects on the gene expression pathway following UV treatment. Indeed, early after UV irradiation, phosphorylation of the translation factor eIF2, which inhibits global translation, occurs very rapidly (Deng et al., 2002; Jiang and Wek, 2005) probably contributing to the initial decrease in protein synthesis. Within the first 6 hours after UV exposure, we also find that the cytoplasmic poly(A) RNA signal also appears to dramatically decrease in intensity (Fig. 4, supplementary material Fig. S6). This is likely to reflect a combination of ongoing or accelerated deadenylation/turnover of cytoplasmic mRNAs, accompanied by decreased transcription (Heine et al., 2008) as we observe early changes in the phosphorylation status of RNA pol II, indicative of reduced transcription, by 6 hours (supplementary material Fig. S7B, Fig. S8). Interestingly, phosphorylation of RNA pol II is further changed after 9 hours (supplementary material Fig. S8), an effect that together with the inhibition of 3' processing known to occur after UV exposure (Mirkin et al., 2008) probably explains why poly(A) RNA does not continue to substantially increase in the nucleus at later times, although mRNA export is blocked.

Although the loss of PABP1 and PABP4 from the cytoplasm could contribute to a reduction in global translation, relocalisation of PABPs is not complete. This might be important in maintaining the translation of specific subsets of cellular mRNAs, consistent with measurable protein synthesis even at the latest time-point (Fig. 3C). Indeed mRNAs that lack secondary structure in the 5'UTR have been shown to have a lower requirement for PABP (Gallie et al., 2000) and other mRNAs appear to contain regulatory elements that increase their capacity to recruit PABP (reviewed by Burgess and Gray, 2010), that could facilitate their translation when PABP levels are low. Therefore, relocalisation of PABPs from the cytoplasm might function analogously to eIF2 $\alpha$  phosphorylation in translational reprogramming. eIF2 phosphorylation causes a global inhibition of translation early after UV treatment, while permitting the continued translation of certain mRNAs important for cell recovery (Powley et al., 2009). However, effects on global translation here are likely to be primarily mediated through PABP1, as knockdown of PABP4 had little effect on protein synthesis (supplementary material Fig. S10), consistent with its relatively low levels in these cells (supplementary material Fig. S1). Alternatively, PABP relocalisation could serve primarily to effect the translation of specific mRNAs, for instance, in releasing the repression of specific mRNAs that are required as part of the recovery process. Indeed, miRNA-mediated repression was recently shown to be sensitive to levels of PABP1 (Walters et al., 2010), and recovery of cell proliferation is observed when

the cells are maintained for longer periods (data not shown). Moreover, the multifunctional nature of PABP1 and our recent work showing that the non-redundant functions of PABP1 and PABP4 are not linked to their roles in global translation (Gorgoni et al., 2011) emphasise the possibility that PABP1 and PABP4 relocalisation could be important in regulating their contribution to other aspects of RNA metabolism.

Although it has been known for some time that PABP1 is imported to the nucleus and, importantly, that this is an energy-dependent process (Afonina et al., 1998), a nuclear role for Pab1 has only been reported in budding yeast (Lemay et al., 2010), which lacks PABPN1. In metazoans, PABP1 interacts with several nucleo-cytoplasmic proteins (e.g. Dizin et al., 2006; Nagaoka et al., 2006) but only the cytoplasmic functions of these interactions have been investigated. Our results indicate that neither PABP1 nor PABP4 is important for bulk RNA export (Fig. 5), suggesting that the energy-dependent import of PABP proteins is not to facilitate this process, although we cannot exclude the possibility that PABP1 and PABP4 may play a more subtle or mRNA-specific role in export, as observed in yeast (Brune et al., 2005; Thakurta et al., 2002). Therefore transit of cytoplasmic PABPs through the nucleus might be primarily to ensure the stability and rapid translation of bound mRNAs as they enter the cytoplasm. In keeping with this, we find that accumulation of PABP in the nucleus is not required for cell viability following UV treatment (Fig. 5). Thus, during stress, the major role of PABP1 and PABP4 relocalisation appears to be their removal from the cytoplasm. PABP knockdown would achieve a similar reduction in cytoplasmic PABP levels, explaining why this manipulation is tolerated after UV exposure (Fig. 5).

Although our mechanistic analysis revealed mRNA-dependent export of PABPs, there also appears to be mRNA-independent PABP export in mammals (Khacho et al., 2008; Woods et al., 2005). However, whereas Pab1 in *S. cerevisiae* is exported independently of mRNA in a process involving interaction of its classical nuclear export signal (NES) with exportin 1, neither mammalian PABP1 nor PABP4 contain a classical NES, and contradictory results exist regarding dependence on Crm1, the homologue of exportin 1 (Khacho et al., 2008; Woods et al., 2005). However, interactions with paxillin (Woods et al., 2005) and translation elongation factor 1 (eEF1) (Khacho et al., 2008) have independently been suggested to be important for PABP1 export by mechanisms that require further definition. Such alternative pathways might contribute to the incomplete relocalisation of PABP following UV exposure and could be targeted for the relocalisation of PABP when bulk mRNA export is unaltered.

The RNA-dependent export of PABP1 uncovered here also explains the observation that transcriptional inhibition, which leads to a cessation of mRNA export (Tokunaga et al., 2006), results in nuclear accumulation of PABP1 (Afonina et al., 1998). In keeping with this, we have found that nuclear export of PABP4 is also sensitive to transcriptional inhibition (supplementary material Fig. S7). Furthermore, PABP1 and PABP4 mutants lacking only the poly(A) RNA binding domains (RRMs 1 and 2) have a partially nuclear localisation consistent with an impaired ability to exit the nucleus bound to mRNA (supplementary material Fig. S9). Our results also provide a mechanism for PABP relocalisation in non HSV-1 infected cells ectopically expressing ICP27 (Dobrikova et al., 2010; Salaun et al., 2010),

showing that this is due to the effects of ICP27 on mRNA splicing, and therefore export (Fig. 6). A block to mRNA export could also explain relocalisation of PABP1 during bunyavirus infection because this is associated with a shut-off of host cell transcription (Thomas et al., 2004). However, our results differ crucially from those of a study on the relocalisation of PABP by the Kaposi's sarcoma-associated herpesvirus (KSHV) protein SOX (shut-off exonuclease), where an induced accumulation of poly(A) in the nucleus was blocked by PABP1 knockdown (Kumar and Glaunsinger, 2010), suggesting that PABP relocalisation in KSHV-infected cells occurs by a distinct mechanism.

In summary, our results suggest that the nucleo-cytoplasmic export of PABPs in metazoans is more similar to yeast than first thought, being mediated through mRNA-dependent as well as mRNA-independent pathways. Because nuclear redistribution of PABP1 has only been reported in a handful of cases, it will be interesting to explore the extent to which the nuclear relocalisation of PABP1 and PABP4 is a strategy for manipulating cellular translation and why certain cellular stresses relocalise PABPs to the nucleus rather than stress granules.

## Materials and Methods

### Plasmids

pEGFPC1 was obtained from Clontech. The shTAP plasmid (pSUPER-TAP) and control plasmid (pSUPER) were kind gifts from Stuart Wilson (Dept. of Molecular Biology and Biotechnology, University of Sheffield, UK) (Williams et al., 2005). ICP27-GFP has been described previously (Salaun et al., 2010).

### Cell culture and treatments

HeLa and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen). 254 nm UV was administered using a Camlab CL-1000 ultraviolet crosslinker. Before treatment the medium was withdrawn and fresh medium was added after irradiation. Mock-treated cells were treated identically except that irradiation was omitted. Arsenite-treated cells were incubated for 1 hour with 0.5 mM sodium arsenite (Sigma) in phosphate-buffered saline (PBS).

### Antibodies

PABP1 and PABP4 rabbit polyclonal antibodies were generated by CovalAB (Cambridge, UK) using a single C-terminal peptide from PABP1 that is identical in human and mouse, and two C-terminal peptides from PABP4 that are also identical in human and mouse. Other antibodies were sourced commercially: G3BP (Becton Dickinson, no. 611126), PARP (Abcam, no. 6079), SC-35 (Abcam, no. 11826), GAPDH (Abcam, no.9484),  $\alpha$ -tubulin (Sigma, no. 6074), RNAPII CTD pS5 (Abcam, no.5131).

### DNA and siRNA transfections

DNA plasmids were transfected using Attractene (Qiagen) diluted in OPTI-MEM (Invitrogen) following the manufacturer's instructions. PABP1 (no. SI00676676) and PABP4 (no. SI04179861) siRNAs or Qiagen AllStars negative control were diluted in OPTI-MEM (Invitrogen) and transfected to a final concentration of 5 nM (10 nM control siRNA for dual PABP1 and PABP4 siRNA transfections) using Hiperfect, following the manufacturer's instructions. siRNA-transfected cells were incubated for 48 hours post-transfection before further treatment or analysis.

### Immunofluorescence

Cells were grown on glass coverslips and fixed in 4% paraformaldehyde (PFA) for 15 minutes and permeabilised for 10 minutes with  $-20^{\circ}\text{C}$  methanol. Samples were incubated with a blocking solution (5% horse serum in PBS) for 1 hour at room temperature before overnight incubation with primary antibodies in the blocking solution at  $4^{\circ}\text{C}$ . The appropriate fluorescently conjugated secondary antibody [anti-rabbit or anti-mouse Alexa Fluor 488 or 546 (Invitrogen)] was added for 1 hour at room temperature at 0.1  $\mu\text{g}/\mu\text{l}$  before mounting the cells in Vectashield containing DAPI (Vector Labs).

Annexin V was detected using a FITC Annexin V Apoptosis Detection Kit I (BD Biosciences). Cells were washed twice in PBS and once in binding buffer, before incubation with 0.5  $\mu\text{g}/\text{ml}$  annexin V-FITC in binding buffer for 15 minutes. Cells were washed once more in binding buffer before fixation.

Fluorescence images were captured using a Zeiss LSM710 confocal laser scanning microscope and Zen 2009 software.

### Fluorescence in situ hybridisation

Cells were fixed and permeabilised as above. Where prehybridisation RNase treatment was applied (Johnson and Sandri-Goldin, 2009), 0.1  $\mu\text{g}/\mu\text{l}$  RNase A in PBS was added to the cells before incubation for 30 minutes at  $37^{\circ}\text{C}$ . Cells were incubated in 70% ethanol for at least 10 minutes, and then 1 M Tris pH 8 for 5 minutes, before addition of a Cy3-conjugated oligo(dT)<sub>40</sub> probe (Eurogentec) in hybridisation buffer (0.005% BSA, 1 mg/ml yeast RNA, 10% dextran sulphate, 25% formamide in  $2\times$  SSC) at 1.3 ng/ $\mu\text{l}$ . Slides were then placed in a humidity chamber for 1 hour at  $37^{\circ}\text{C}$ . Following hybridisation, cells were washed once with  $4\times$  SSC and once with  $2\times$  SSC. For immediate visualisation, cells were washed once with  $2\times$  SSC + 0.1% Triton X-100 before mounting. For additional immunofluorescence after in situ hybridisation, primary antibodies were diluted in  $2\times$  SSC + 0.1% Triton X-100 (with the exception of anti-PABP1, which was diluted in  $1\times$  SSC + 0.1% Triton X-100) and incubated for 1 hour at room temperature. Cells were then washed in  $2\times$  SSC (or  $1\times$  SSC + 0.1% Triton X-100 after anti-PABP1 staining) three times and the appropriate secondary antibody (0.1  $\mu\text{g}/\mu\text{l}$ ) was applied, diluted in  $2\times$  SSC + 0.1% Triton X-100. Cells were incubated for 1 hour at room temperature before three final washes in  $2\times$  SSC ( $1\times$  SSC + 0.1% Triton X-100 after anti-PABP1 staining) and were mounted and examined as described above.

### Western blotting

Cell extracts were prepared in RIPA buffer [50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.2% SDS, 10 mM sodium pyrophosphate, 25 mM glycerol-2-phosphate, 0.5% deoxycholate, 100 mM sodium orthovanadate, 5 mM NaF, 2 mM dithiothreitol, protease inhibitor tablets (Roche); pH 7.4]. Equivalent amounts of protein were resolved on Invitrogen NuPAGE 4–12% MOPS gels and transferred to polyvinylidene fluoride membranes. Membranes were subject to western blotting with primary antibodies, PABP1 (1:10,000), PABP4 (1:5000), GAPDH (1:1000), PARP (1:1000),  $\alpha$ -tubulin (1:10,000), G3BP (1:2000) or RNAPII CTD pS5 (1:2000), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit (Sigma #A0545-1; 1:100,000) or goat anti-mouse (Pierce #31444; 1:20,000) secondary antibodies and developed using an enhanced chemiluminescence reagent (Amersham).

### [ $^{35}\text{S}$ ]methionine labelling

Cells cultured in six-well plates were labelled for 15 minutes with 50  $\mu\text{Ci}/\text{ml}$  [ $^{35}\text{S}$ ]methionine (MP Biomedicals) in methionine-free minimal essential medium (MP Biomedicals). After washing with PBS, cell extracts were prepared in RIPA buffer and protein concentration determined by the Bradford assay (Bio-Rad). Incorporation of  $^{35}\text{S}$  into samples was measured by trichloroacetic acid precipitation of equal amounts of total protein on glass microfibre filters, and liquid scintillation counting in OPTIPHASE solution (Perkin Elmer) using a 1450 microbeta Wallac Trilux liquid scintillation counter. Statistical significance was determined by paired Student's *t*-tests between untreated samples and samples at various time points after treatment, using GraphPad.  $P < 0.05$  was considered statistically significant.

### Annexin V/PI staining and flow cytometry

For cell growth assessment, HeLa cells were grown in six-well dishes and harvested by trypsinisation at the appropriate time following mock or UV treatment. DMEM supplemented with 10% fetal bovine serum was added following trypsinisation and cells were fixed in a final concentration of 4% PFA. Flow-check fluorospheres (Beckman Coulter) were added at a final concentration of 200,000 spheres/ml cells before analysis by flow cytometry, and used to calculate the number of cells/ml using Cell Quest Software. All manipulations were performed in triplicate. Annexin V/PI staining was conducted using BD Biosciences FITC annexin V Apoptosis Detection Kit I following manufacturer's instructions. Briefly, HeLa cells were harvested by trypsinisation, collected by centrifugation and resuspended in the supplied binding buffer at a concentration of  $\sim 10^6$  cells/ml. To 100  $\mu\text{l}$  of cells, 10  $\mu\text{l}$  of a 10  $\mu\text{g}/\text{ml}$  annexin V-FITC solution was added, before incubation on ice for 15 minutes. 10  $\mu\text{l}$  of PI solution was then added to the cells followed by a further 400  $\mu\text{l}$  binding buffer before analysis by flow cytometry. Measurements of 10,000 cells were made using Cell Quest Software. Statistical analysis of the percentage of labelled cells was performed using GraphPad Prism. Statistical significance was determined by analysis of variance (ANOVA) with Bonferroni post-tests between experimental groups (GraphPad).  $P < 0.05$  was considered statistically significant.

### DNA damage analysis

DNA damage was assessed using an OxiSelect UV-Induced DNA Damage ELISA Kit (CPD Quantitation; Cell Biolabs Inc., San Diego, CA) on DNA prepared with a Qiagen Blood Kit, according to the manufacturer's instructions except where 6-



4PP antibodies (Cosmo Bio Co. Tokyo, Japan) were substituted for CPD antibodies to allow measurement of both lesions.

#### Quantification of relative PABP levels

pET-hPABP1 was described previously (Smith et al., 2011). pET-hPABP4 was created by PCR from IMAGE clone no. 30331896 using primers 5'-AGGCTTC-TATGAACGCTGCGGCCAGC-3' and 5'-CTCGAGAGAGGTAGCAGCAGCA-ACAGCG-3' and insertion of the product into *Xho*I and *Hind*III sites of pET28c(+) (Novagen). Human PABP1 and PABP4 proteins were expressed, purified and quantified as described previously (Smith et al., 2011). Western blotting of purified proteins and HeLa cell extracts was conducted using an Alexa Fluor 680 nm conjugated anti-rabbit secondary antibody (Invitrogen A21076) and scanned using a Licor Odyssey 9120 infrared imager.

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